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Control of blue mold decay of apple during commercial controlled atmosphere storage with yeast antagonists and sodium bicarbonate☆

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ABSTRACT

A mixture of two yeast antagonists, *Metschnikowia pulcherrima* and *Cryptococcus laurentii*, originally isolated from apples and exhibiting greater biocontrol activity against blue mold of apple than either yeast applied alone, were used in combination with sodium bicarbonate (SBC) in a pilot test in which treated fruit were stored under commercial controlled atmosphere (CA) storage conditions. Conidia of *Penicillium expansum*, antagonists cells and SBC were added to the drench solution. The treatments were applied to apples by drenching entire bins filled with apples containing 100 wounded fruit evenly distributed among five positions in each bin. The treated fruit were stored in commercial CA storages for approximately six months in the 2005–2006 and 2006–2007 storage seasons and then evaluated for incidence of decay. In both years, the treatments with the antagonist alone or in combination with SBC were equally effective and reduced blue mold incidence by 84–97% in 2005–2006 and 73–82% in 2006–2007. SBC alone significantly reduced blue mold incidence compared to the non-treated control but was less effective than the antagonist alone or in combination with SBC. This pilot test showed that the combination of these two antagonists and SBC can be an effective decay control method under commercial CA conditions, confirming results from our earlier laboratory studies using similarly treated fruit stored under CA conditions.
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1. Introduction

Biological control of postharvest diseases has made great advances, especially during the past decade, during which the usefulness of this approach has been proven under commercial conditions (Janisiewicz and Korsten, 2002). The commercial products Aspire™ (Ecogen, Inc., Langhore, PA) based on the yeast *Candida oleophila* (Droby et al., 1993, 1998) and BioSave™ 100 and 110 (JET Harvest Solutions, Longwood, FL) containing saprophytic strains of *Pseudomonas syringae* (Janisiewicz and Jeffers, 1997; Janisiewicz and Marchi, 1992) were registered by the United States Environmental Protection Agency for application to pome and citrus fruits in 1995. The use of BioSave™ has been continually increasing and the original registration for postharvest application to apples, pears and citrus fruit has been extended to cherries, potatoes, and sweet potatoes (Buckner, 2005; Holmes and Edmunds, 2005; Stockwell and Stack, 2007). On pears it was reported to be the most effective postharvest treatment in integrated management trials and was comparable to or better than a standard fungicide (Sugar, 2006). Although this product has been very effective in various systems and under a variety of conditions, as with any biocontrol agent, it has its limitations, especially those imposed by environmental conditions. Other biocontrol products for postharvest application that are on the market include YieldPlus™ (Anchor Yeast, Cape Town) containing *Cryptococcus albidus* (De Koch, 1998) in South Africa, and Shemer™ (AgroGreen, Asgdod) containing *Metschnikovia fructicola* in Israel (Kurtzman and Droby, 2001; Karabulut et al., 2002, 2003). Both are registered in their respective countries for control of postharvest decays on several fruits including grapes, pome, stone, and citrus fruit. AvoGreen™, containing *Bacillus subtilis*, is registered in South Africa for orchard application to control postharvest anthracnose of avocado (see Janisiewicz and Korsten, 2002). Unfortunately, there are no reports on the extent of use of these products.

In selecting yeast biocontrol agents for controlling postharvest decays of pome fruits, we focused on yeasts commonly occurring on apple and in apple cider because they have been consumed by

☆ Mention of trade names or commercial products in the publications solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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humans for a long period of time without any indication of adverse health effects. A long-term history of consumption by humans would aid registration of these organisms. One such organism is *Metschnikowia pulcherrima*, a yeast which has been reported to occur commonly on apple and in apple cider (Bizeau et al., 1990; Beach, 1958, 1993; Clark et al., 1954; Davenport, 1976) and which is known to control various postharvest decays on pome fruits and grapes (De Curtis et al., 1996; Janisiewicz et al., 2001; Nigro et al., 1999; Piano et al., 1997; Sapardo et al., 2002). This yeast is phenotypically and genetically very diverse. Strains of this yeast isolated from a single orchard differed greatly in their tolerance to chemicals used after harvest, ability to grow at a low (1 °C) storage temperature, and in their effectiveness against blue mold on apples (Janisiewicz et al., 2001). *Cryptococcus laurentii* is another yeast commonly found on apple and in apple cider (Bizeau et al., 1990; Davenport, 1976; Williams et al., 1956), and on many other fruits (Dennis, 1976). It can reduce postharvest decays on many fruits including blue mold caused by *Penicillium expansum* and gray mold caused by *Botrytis cinerea* of apple (Lima et al., 1998; Roberts, 1990); gray mold of pear, strawberries, kiwifruit, and table grapes (Chand-Goyal and Spotts, 1997; Lima et al., 1998; Sugar and Spotts, 1999; Zhang et al., 2005); *Rhizopus* rot of strawberries and peach (Zhang et al., 2004, 2007) and *P. expansum* decay on jujube fruit (Qin and Tian, 2004). These yeast antagonists can be very effective in reducing fruit decays but their effectiveness, as with any biocontrol agent, may decline under suboptimal conditions. For example, exposure to chemicals used after harvest, such as antioxidants or flotation salts, may weaken the antagonist and reduce its survival, resulting in less decay control (Janisiewicz et al., 2001). Biocontrol may also be less effective on more mature apples with lower resistance to decay, and higher concentrations of the antagonist may be needed to compensate for this reduced resistance to achieve an acceptable level of control (Janisiewicz, unpublished). The use of antagonist mixtures can reduce variability, increase efficacy of biocontrol agents and improve control of fruit decays (Janisiewicz, 1996). The application of yeast mixtures of *Cryptococcus infirmo-miniatus* with *C. laurentii* was as effective as fungicide (TBZ, thiabendazole) treatment in controlling postharvest diseases on pears (Chand-Goyal and Spotts, 1997), and the mixture of *Rhodotorula glutinis* and *C. albidus* (Calvo et al., 2003) was more effective than the individually applied yeasts against gray mold on apples. Mixtures of yeasts with bacterial antagonists, e.g., *Sporobolomyces roseus* with *P. syringae* (Janisiewicz and Bors, 1995) and *Candida sake* with *Pantoea agglomerans* (Nunes et al., 2002) also were more effective than the individual antagonists in controlling blue mold on apples. Combining antagonists with other treatments that are alternatives to conventional fungicides such as substances generally recognized as safe (GRAS), or physical treatments such as heat, UV or microwave irradiation can further improve decay control (Leverentz et al., 2000; Smilanick et al., 1999; Wilson et al., 1994; Zhang et al., 2004; Porat et al., 2002). In our earlier study, integrating biocontrol by *M. pulcherrima* with heat treatment and sodium bicarbonate, a GRAS substance, significantly improved control of blue mold on 'Golden Delicious' apples (Conway et al., 2004), and a mixture of *M. pulcherrima* with *C. laurentii* combined with these treatments further improved control (Conway et al., 2005). A laboratory-scale test under either air or controlled atmosphere (CA) storage showed additive effects of this antagonist mixture, sodium bicarbonate, and CA storage on the reduction of blue mold on 'Golden Delicious' apples (Conway et al., 2007).

The objective of this study was to determine the effectiveness of the combined treatments of the antagonist mixture of *M. pulcherrima* and *C. laurentii*, and sodium bicarbonate in controlling blue mold of 'Golden Delicious' apples in a pilot test using bin drenching application followed by commercial CA storage conditions.

2. Materials and methods

2.1. Pathogen

The *P. expansum* isolate (MD-8) used in this study is an aggressive pathogen isolated from a decayed apple. The pathogen was grown on potato dextrose agar (PDA) and virulence was maintained by periodic transfers through apple fruit. Aqueous suspensions of the conidia were prepared in 50-mL tubes from a ten-day-old culture as previously described (Janisiewicz and Marchi, 1992). The conidial suspension was prepared as a concentrated stock which, after addition to 125 L of water in the drencher tank, resulted in a final conidial concentration of 3×10^6 L^{-1}.

2.2. Antagonists

The antagonists used were the yeast *M. pulcherrima* strain FMB-24H-2, capable of growing at cold temperatures (Janisiewicz et al., 2001), and *C. laurentii* strain ST4-E14, both isolated from wounded apple in an unmanaged orchard. The yeasts were grown in 200 mL of nutrient yeast dextrose broth (NYDB) medium in 500-mL Erlenmeyer flasks on a rotary shaker at 200 rpm at 26 °C for 24 h. The cells were then harvested by centrifugation at 7000 × *g* for 10 min and the pellet was collected and stored in 50-mL tubes at 4 °C. The yeasts were produced over a two-week period preceding their application to fruit. On the day of application, equal weights of the fresh cell preparations of each yeast were resuspended in 1 L of water to make a concentrated stock suspensions which, after adding to the drench tank with 125 L of water, resulted in yeast cell concentrations of approximately 1.2×10^{10} L^{-1} (according to the standard curves developed for fresh yeast cell preparations). Then 0.5 L of each yeast suspension was combined to obtain a concentrated antagonist mixture that was added to the drencher tank.

2.3. Sodium bicarbonate

Sodium bicarbonate (SBC) (Pure Baking Soda, Arm & Hammer, Princeton, NJ) was used at a concentration of 2% (weight/volume). The appropriate amount of SBC was added to the drench tank and the suspension was circulated for at least 10 min before adding yeast mixture and/or pathogen and drenching the fruit.

2.4. Fruit

Apples were harvested in a commercial orchard in Biglerville, PA, in 2005, and in an experimental orchard at the Virginia Polytechnic Institute & State University (VPI&SU) Experiment Station in Winchester, VA, in 2006, when a majority of the fruit in the test cultivar reached a commercially acceptable stage of maturity for controlled atmosphere cold storage based on flesh firmness, soluble solids concentration and starch index (SI) rating. Ten apples were harvested at random and flesh firmness measured on opposite sides of each fruit with a penetrometer (Model FT-327; McCormick Fruit Tech, Yakima, WA) fitted with an 11.1-mm Magness-Taylor probe mounted in a drill press stand. Soluble solids concentration was determined with a digital refractometer (Atago PR100; NSG Precision Cells, Inc., Farmingdale, NY) from a composite juice sample from the ten-apple sample. The starch–iodine index was visually rated using the Cornell generic starch scale 1–8 (Blanpied and Silsby, 1992). Apples were considered acceptable for harvest when the average starch index rating was 3–5. The fruit were harvested into standard fruit bins (1.06 m wide × 1.22 m long × 0.79 m high), each holding about 400 kg of apples.

2.5. Fruit treatment

To treat fruit, bins with fruit were half-emptied, and 100 fruit wounded with the point of a 9-cm common wire nail to a depth of 3 mm were placed midway between the bottom and top of the bin in the center and near each of the four corners (20 fruit in each location) and covered with apples to fill the bin. A thin bird netting (eye 2.54 cm × 2.54 cm) was placed below and over the wounded fruit to facilitate easy separation of the wounded fruit from the rest of the fruit for evaluation of decay incidence after storage. The bins were drenched with treatment suspensions using a portable drencher having a 150-L capacity reservoir (Janisiewicz et al., 2005). The travel time of a bin through the drencher was 2 min. During that time 172 L of drenching suspension passed through the bin. The first treatment was *P. expansum* alone. This was followed by *P. expansum* + SBC drench, *P. expansum* + antagonist mixture drench, and *P. expansum* + SBC + antagonist mixture drench. After each treatment application, the drencher and hoses were emptied and washed with water extensively. There were three bins of apples per treatment and each bin constituted a single replicate. Bins with treated fruit were placed in CA (1.5 kPa O_2, 2.0 kPa CO_2) storage at ~1 °C and wounded fruit were removed from storage and evaluated for decay incidence after 5.5 months.

2.6. Recovery of the antagonist

The populations of *C. laurentii* ST4-E14 and *M. pulcherrima* FMB-24H-2 were determined after storage in CA at 1 °C for 5.5 months in 2007. From each bin (replicate) receiving the antagonist treatment, antagonists were recovered from wounds of five apples, one sample from each of the five wound sites of the apples within a bin as previously described (Conway et al., 2000). Briefly, the wounded area was removed to a depth of 1 cm with a cork borer (1 cm dia.), placed in Stomacher bags with 4.5 mL of sterile distilled water, extracted with a Stomacher blender (Stomacher 80; Seward Medical, London) at normal speed for 2 min, filtered through glass wool, diluted 1:100 and plated on NYDA medium with a Autoplate 400 (Spiral Biotech, Inc., Norwood, MA) set to the uniform deposition. The colonies were counted after 3 days storage at 24 °C. The antagonists were distinguished morphologically based on the color of the colonies with *C. laurentii* being white and *M. pulherrima* light pink with characteristic dark centers on the reverse side of the plate.

2.7. Statistical analysis

Results from the 2005–2006 and 2006–2007 experiments were analyzed using the general linear model (GLM) of the Statistical Analysis System (SAS® Version 9.1, 2004). To correct variance heterogeneity, the values were arcsine transformed for the analysis. A Waller-Duncan multiple range test ($P = 0.05$) was conducted to separate means of the fruit decay values for individual treatments in both storage seasons. The expected additive effect of the treatments was calculated according to formula: $E(\exp) = a + b - (ab/100)$, where E is the expected additive effect, and a and b are percent of decay reduction by the individual treatments relative to the control (Levy et al., 1986). The means for recovery of the antagonists' populations from apple wounds after 5.5 months in CA storage in 2006–2007 storage season were separated using the t-test.

3. Results and discussion

After 5.5 months of CA storage of apples in the 2005–2006 season, differences in decay incidence among the treatments were clearly visible ($F = 46.6$, $P = 0.0001$). The greatest amount of decay occurred on the control treatment (*P. expansum* alone) followed by SBC alone, and then the antagonist mixture alone or in combination with SBC ($P = 0.05$) (Fig. 1). While differences between the antagonist-containing treatments were not significant, there was a clear tendency toward a lower incidence of decay on fruit treated with the combination of the SBC and antagonist mixture. The combination of the SBC and antagonist mixture reduced decay by 97.4% compared to the *P. expansum* drench alone. This was 7.8% better than the randomly expected additive effect (89.6%) calculated from the decay reduction by the individual treatments. The differences in incidence of decay on apples stored in the 2006–2007 season ($F = 78.5$, $P = 0.0001$) followed the same pattern as those stored in 2005–2006, but overall there was a higher incidence of decay. The combination of the SBC and antagonist mixture reduced decay by 66.7%. This was 14.9% less than the randomly expected additive effect (81.6%, 2006–2007 season) calculated from the decay

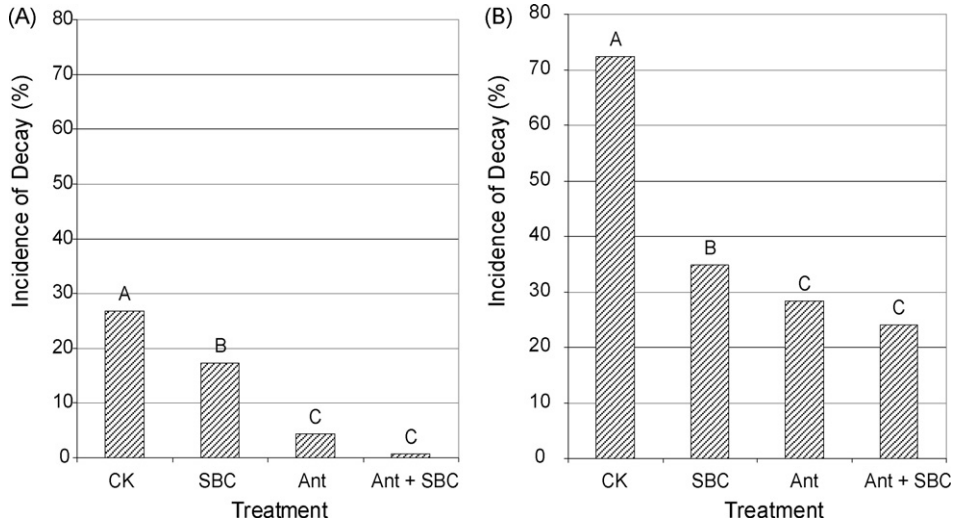

Fig. 1. Incidence of decay on 'Golden Delicious' apples drenched with *Penicillium expansum* conidia alone (CK) or in combination with sodium bicarbonate (SBC), a mixture of *Metschnikowia pulcherrima* and *Cryptococcus laurentii* antagonists (Ant) or the antagonist mixture + sodium bicarbonate (Ant + SBC) using a portable drencher in (A) 2005–2006, and (B) 2006–2007 storage seasons. The treated apples were placed in controlled atmosphere (CA) storage for 5.5 months and then evaluated for incidence of decay. Within plots, bars with different letters are significantly different according to Waller-Duncan multiple range test ($P = 0.05$).

reduction by the individual treatments. Nevertheless, the two years average reduction in decay by the combination of SBC and antagonist mixture (82.1%) was very close to the predicted additive value (84.6%), suggesting an overall additive effect of these treatments across years of treatment. Analysis of the data combined from both storage seasons resulted in the same mean separation as from the individual seasons.

The reduction of decay by SBC alone was similar to our earlier results from the drencher application of SBC to 'Red Delicious' apples, stored in regular air storage for ~3 months, where the application of SBC alone reduced decay by one half (Janisiewicz et al., 2005). In that experiment, the reduction of decay on 'Golden Delicious' fruit was much greater (from 33.1% incidence on the *P. expansum* treatment alone to 2.7% on *P. expansum* + SBC-treated fruit) but overall the incidence of decay was lower due, most likely, to the high natural resistance of 'Golden Delicious' fruit to blue mold decay. While the percent reduction of blue mold decay by treating pathogen-inoculated apples with SBC alone varied in experiments under laboratory conditions, its use in combination with the antagonists always resulted in an additive effect (Conway et al., 2005, 2007). The greater effectiveness of the antagonist mixture compared to SBC also confirmed the results from our laboratory experiments (Conway et al., 2007).

In the 2005–2006 season, the fruit had a good appearance and there were no signs of shriveling or any other superficial disorders. The storage conditions in the packinghouse used in the 2006–2007 season were less than optimal when compared to the storage used in the 2005–2006 season as evidenced by the shriveled appearance of some of the fruit. The treatments did not negatively affect fruit appearance in either season.

Populations of *M. pulcherrima* in apple wounds appear to be slightly higher than those observed in the earlier experiments under laboratory conditions after 4 months in CA storage. Recovery of $\log_{10}$ CFU wound^{-1} was 6.2 in the laboratory experiments (Conway et al., 2007) versus 6.7 in the pilot test in 2006–2007. When applied with SBC, recovery was 6.1 in the laboratory experiments (Conway et al., 2007) and 7.0 in the pilot test. However, unlike the laboratory experiments, the differences between populations in the control treatment (*P. expansum* alone) and the *P. expansum* + SBC treatments were statistically significant in the pilot tests ($P = 0.05$). There were no differences in recovery of *C. laurentii* populations applied to the fruit in the control (5.3 of $\log_{10}$ CFU wound^{-1}) or SBC (5.5 $\log_{10}$ CFU wound^{-1}) treatments in the pilot test, although the populations were about one $\log_{10}$ unit lower than in the laboratory experiments. The low recovery of *C. laurentii* from the pilot-test fruit in 2007 may have resulted from the effect of less than optimal storage conditions to which the fruit (and the antagonist) were exposed. This includes an apparent lower humidity that may have had a deleterious effect on antagonist survival due to greater desiccation as indicated by fruit shriveling. This would result in a more rapid concentrating effect of SBC, which may have been toxic to this yeast.

The results of this pilot test indicate that the combination of the mixture of *M. pulcherrima* and *C. laurentii* antagonists with SBC can be very effective in controlling blue mold of apples under commercial CA conditions, confirming results from our earlier laboratory experiments (Conway et al., 2007). This pilot test also showed that even under suboptimal storage conditions, which resulted in shriveling of the fruit, decay could be reduced by 66.7%. In our previous laboratory experiments, the combination of SBC with *M. pulcherrima* alone or mixed with *C. laurentii* always had a smaller average incidence and/or severity of blue mold decay than any of those treatments alone, although those differences were not always statistically significant, especially when little decay developed on the treated fruit (Conway et al., 2005, 2007; Janisiewicz et al., 2005). This antagonist mixture is also compatible with a heat treatment (4 days at 38 °C) which has the advantage of having eradicative activity (Conway et al., 2005). Results from our earlier work and the pilot test indicate the compatibility of biological control with other alternative treatments to synthetic fungicides such as sodium bicarbonate, heat, or CA storage, and the additive effect of such combinations for protecting fruit under commercial storage conditions.

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